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(54) Title: IMMUNOSUPPRESSION OF THE HUMORAL IMMUNE RESPONSE BY ANTI-CD20 ANTIBODIES

(57) Abstract: The present invention provides methods for reducing or preventing a patient's humoral immune response from developing antibodies that neutralize or limit the efficacy of therapeutic molecules. The methods are based on the use of anti-CD20 antibodies to deplete antibody-producing B-cells in a patient. Suitable dosage and therapeutic regiments are also provided. The present invention further provides kits with reagents for practicing the disclosed methods.

IMMUNOSUPPRESSION OF THE HUMORAL IMMUNE RESPONSE BY ANTI-CD20 ANTIBODIES

This application claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 60/290,319, filed May 11, 2001, which is incorporated by reference herein in its entirety.

1. FIELD OF THE INVENTION

The present invention relates to methods for reducing or preventing the production of endogenous antibodies to an immunogenic molecule that is administered as a therapeutic agent or a component of a therapeutic composition, thereby minimizing unwanted side effects to administration of the immunogenic molecule and maximizing its efficacy as a therapeutic or the efficacy of a therapeutic associated with it. Said methods entail the depletion of antibody-producing B-cells in the patient by administering an anti-CD20 antibody prior to, concurrently with or after administration of the immunogenic molecule to the patient.

2. BACKGROUND OF THE INVENTION

The immune system of vertebrates (for example, primates, which include humans, apes, monkeys, *etc.*) consists of a number of organs and cell types (lymphocytes) which have evolved to accurately and specifically recognize antigens, for example proteins of foreign microorganisms which invade the vertebrate-host, specifically bind to such foreign antigens and eliminate/destroy such foreign microorganisms. B-cells are a type of lymphocyte responsible for antibody production (humoral immunity). Each B cell within the host expresses a different antibody on its surface--thus, one B cell will express an antibody specific for one antigen, while another B cell will express an antibody specific for a different antigen. Accordingly, B-cells are quite diverse, and this diversity is critical to the immune system. In humans, each B cell can produce 10^7 to 10^8 antibody molecules. B cells which express a particular antibody multiply by clonal expansion. When stimulated by cytokines, B-cells differentiate into plasma cells, which have a surplus of protein-producing endoplasmic reticulum (ER). The plasma cells have a short life cycle, and die when the foreign antigen has been neutralized. The responding B-cell population is subsequently reduced to its normal size apart from a few cells remaining as memory cells.

Antibodies or immunoglobulins (Ig) are proteins composed of heavy chains and light chains, each of which contains a constant region and a variable region. Antibodies bind specifically to antigens and form a complex, which may cause agglutination and precipitation, mask the active sites of the antigens thereby neutralizing its activity, or activate the complement cascade. A single Ig complexed with its antigen can activate a complement cascade with mobilization of up to 10^9 new complement molecules carrying enzymes that rapidly lyse antigen-carrying cells. The most abundant antibody subtype is IgG, which has a high antigen affinity and is the antibody of the secondary response to protein antigens (*e.g.*, viruses and toxins).

Like other cells of the immune system, B-cells express cell surface proteins which can be utilized as "markers" for differentiation and identification. One such human B cell marker is the human B lymphocyte-restricted differentiation antigen CD20 (also known as B1 or Bp35). CD20 is initially expressed during early pre-B cell development and remains until plasma cell (antibody-producing cell) differentiation. Specifically, the CD20 molecule may regulate a step in the activation process which is required for cell cycle initiation and differentiation.

The humoral immune response, which is beneficial for preventing infectious diseases by neutralizing the disease agents, has been a limiting factor in the development of various protein therapeutics due to the neutralization of therapeutic proteins by antibodies. For example, the efficacy for immunotoxins as therapeutic agents has been severely hampered by the humoral immune response. One of the main obstacles to overcome with regard to immunotoxins is the immunogenicity of the toxin component. Patients treated with immunotoxins rapidly develop their own antibodies against one or both portions of the immunotoxin molecule, often within 10 days to two weeks of starting immunotoxin therapy (Pai and Pastan, 1993, JAMA 269:78-81; Skolnick, 1993, JAMA 270:2280; Reiter and Pastan, 1998, Trends Biotechnol.16(12):513-20; Thrush *et al.*, 1996, Ann. Rev. Immunol. 14:49-71), which can limit therapeutically beneficial administration of immunotoxin to as little as a 10-day period (Reiter and Pastan, 1998, Trends Biotechnol.16(12):513-20). A summary of 15 clinical trials with immunotoxins in which antibody induction was studied determined that in 12 (80%) of the trials, at least 50% of patients developed antibodies against the immunotoxin being evaluated (Vitetta, *et al.*, 1993, Immunology Today 14:252-259). In four of the clinical trials more than 90% of the patients developed antibodies.

Immunotoxin treatment needs to be given repeatedly in order to maximize tumor regression and eradication (Friedman, *et al.*, 1993, 150:3054-61; Skolnick, JAMA

270:2280; and Wawrzynczak, 1991, Br. J. Cancer 64:624-630). When endogenous antibody formation occurs, the efficacy of immunotoxin treatment is substantially diminished or negated. This decreased efficacy is thought to result from the increased rate of clearance of the immunotoxin or from blocking of the receptor site or toxic activity site of the immunotoxin (Vitetta, *et al.*, 1993, Today 14:252-259; and Wawrzynczak, and Derbyshire, 1992, Immunology Today 13:381-383). This limits the utility of administering serial, repeated doses, and thus limits the potential efficacy of immunotoxins. Thus, there is a need for methods that will limit a patient's humoral immune response to therapeutic agents such as immunotoxins or other immunogenic proteins, so that the efficacy of the therapeutic agents can be maximized.

Various approaches to overcome the problem of an immune response that abrogates the effect of a therapeutic agent have been tried but without success. Immunosuppressive drugs such as cyclophosphamide, prednisone, azathioprine and cyclosporin A failed to prevent patients from developing endogenous antibody in the face of repeated administration of immunotoxin (Wawrzynczak, 1991, Br. J. Cancer 64:624-630). One immunosuppressive agent, CTLA4 Ig, when co-administered with an immunotoxin, inhibited the production of antibodies against the toxin in rodents and dogs; however, this reagent has not been approved for human use and thus not tested for its efficacy in reducing the humoral response to immunotoxins in humans (Siegall *et al.*, 1997, J. Immunol. 159(10):5168-73).

Alternatively, approaches to reduce immunogenicity have been explored. For example, in experimental animals, modification of a *Pseudomonas* exotoxin-derived immunotoxin with monomethoxy-polyethylene glycol (mPEG) diminished immunogenicity 5- to 10-fold, prolonged circulation time and preserved its anti-tumor effect (Wang *et al.*, Cancer Research 53: 4588-94); however, this type of modification has not been commercially developed for immunotoxins. Humanizing murine antibodies has been seen as a possible solution to the problem of immunogenicity (Skolnick, 1993, JAMA 270:2280; and Winter and Harris, 1993, Immunol. Today 14:243-246). However, this only affects the monoclonal antibody portion of the immunotoxin and does not alter the immunogenicity of the toxin component. Aside from monoclonal antibodies, the current art has not solved the immunogenicity problem associated with proteinaceous therapeutic agents such as toxin components of immunotoxins. Thus, methods of minimizing or abolishing the immunogenicity of immunogenic molecules or the host immune response thereto in patients is still needed.

Citation or identification of any reference herein shall not be construed as an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention provides methods for reduction or prevention of an immune response to an immunogenic molecule in a patient, with the proviso that the immunogenic molecule is not a component of a tissue transplant or an autoantigen, comprising administering to a patient an anti-CD20 monoclonal antibody in an amount sufficient to deplete the patient's circulating B-cells, wherein said anti-CD20 monoclonal antibody is administered to the patient prior to, concurrently with or after the immunogenic molecule is administered to the patient.

In certain embodiments, the immunogenic molecule is a therapeutic molecule. In one embodiment, the immunogenic molecule is an immunotoxin. In another embodiment, the immunogenic molecule is a fusion protein. In one mode of the embodiment, the fusion protein comprises a toxin. In another mode of the embodiment, the fusion protein comprises an enzyme such as a pro-drug converting enzyme. In another embodiment, the therapeutic composition comprises a gene therapy vector.

In certain embodiments of the invention, the anti-CD20 monoclonal antibody is a chimeric antibody. In one embodiment, the chimeric anti-CD20 antibody is chimeric 2B8 antibody. In another embodiment, the chimeric anti-CD20 antibody is chimeric B1 antibody. In another embodiment, the anti-CD20 monoclonal antibody is a humanized antibody. In yet another embodiment, the anti-CD20 monoclonal antibody is a human antibody.

In certain specific embodiments, the amount of anti-CD20 antibody administered is sufficient to deplete the patient's circulating B-cells by at least 25%, by at least 35%, by at least 50%, by at least 60%, by at least 75%, by at least 85%, or by at least 90%. Preferably, said depletion is achieved prior to the administration of the immunogenic molecule to the patient.

In preferred embodiments of the invention, the immunogenic molecule is administered to the patient as a therapeutic or as a component of a therapeutic composition. In one embodiment, the anti-CD20 antibody is administered concurrently with the immunogenic molecule. In other embodiments, the anti-CD20 antibody is administered prior to administration of the immunogenic molecule. In one embodiment, the immunogenic molecule is administered within two weeks of administration of the anti-CD20 antibody. In another embodiment, the immunogenic molecule is administered within

ten days of administration of the anti-CD20 antibody. In another embodiment, the immunogenic molecule is administered within one week of administration of the anti-CD20 antibody. In another embodiment, the immunogenic molecule is administered within five days of administration of the anti-CD20 antibody. In yet other embodiments, the immunogenic molecule is administered within three or two days of administration of the anti-CD20 antibody.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Inhibition of protein synthesis in L2987 human carcinoma cells by serum samples of patients receiving BR96 sFv-PE40 is reduced by the present human anti-toxin antibody (HATA) present in the serum. The X axis shows the dilution of each serum sample (□ corresponds to a sample with a HATA titer of 1:21,870; Δ corresponds to a sample with a HATA titer of 1:590,490; and x corresponds to a sample with a HATA titer of 1:90) or the BR96 sFv-PE40 control (◆). The X axis shows the dilution of the serum and the Y axis shows the percentage inhibition of protein synthesis relative to untreated L2987 human carcinoma cells. The data indicate that neutralization of BR96 sFv-PE40 activity by HATA reduces its toxicity and therefore its inhibition of protein synthesis in the L2987 cell line relative to the BR96 sFv-PE40 control. The extent of neutralization correlates with the HATA titer.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for using anti-CD20 monoclonal antibodies to limit or prevent an immune response to an immunogenic molecule in a patient, with the proviso that the immunogenic molecule is not a component of a tissue transplant or an autoantigen. The immunogenic molecule can be a monoclonal antibody, a toxin, a pro-drug, an enzyme, a gene therapy vector, or a combination of the foregoing.

5.1 ANTI-CD20 ANTIBODIES

The present invention encompasses the use of anti-CD20 antibodies to deplete a patient's peripheral B lymphocyte population, so that a therapeutic agent can be administered to the patient without eliciting an immune response against the therapeutic that would compromise its efficacy.

Any human, humanized or chimeric anti-CD20 antibody can be employed in the methods of the invention. In a highly preferred embodiment, the anti-CD20 antibody

comprises the variable region or the CDRs of monoclonal antibody 2B8. In a preferred mode of the embodiment, the anti-CD20 antibody is chimeric 2B8 antibody ("C2B8").

C2B8 is commercially available as RITUXAN®, and has been approved by the FDA for the treatment of patients with relapsed or refractory, low grade or follicular, CD20-positive, B-cell non-Hodgkin's lymphoma (NHL). C2B8 is an IgG1κ immunoglobulin with murine light- and heavy-chain variable region sequences. C2B8 comprises 2 heavy chains of 451 amino acids each and two light chains of 213 amino acids each, with a total molecular mass of 145kD. C2B8, which has a binding affinity to CD20 of about 8nM, induces lysis of B-cells to which it binds. *In vitro* studies indicate that this occurs through a variety of mechanisms: through antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and induction of apoptosis (programmed cell death). Administration of C2B8 results in rapid depletion of both normal and malignant B-cells, and recovery begins at approximately one to nine months following completion of treatment, depending on the dose administered (*see* Maloney, 1999, Semin Oncol. 26(5 Suppl 14):74-8).

In yet another mode of the embodiment, the C2B8 antibody is glycosylated with bisected oligosaccharides. Glycosylation of C2B8 by recombinant expression in CHO cells that concurrently expressed GnTIII, an enzyme which catalyzes the formation of bisected oligosaccharides, yielded an antibody with higher *in vitro* ADCC activity than unmodified C2B8 (Jean-Mairet *et al.*, 2000, abstract no. 698; International Society for Preventive Oncology Meeting 2000).

In other embodiments, the anti-CD20 antibody comprises the variable region or the CDRs of one or more of the following anti-CD20 monoclonal antibodies: FB1, 1F5, 2H7, 93-1B3, 109-3C2, B1, B9E9, 7D1, H147, L26, L27, and MEM-97.

Many of these anti-CD20 antibodies have been administered to humans in clinical trials and been deemed safe for human use. For example, radio-iodinated B1 has been used in the treatment of B-cell lymphoma (*see, e.g.*, Kaminski *et al.*, 1993, New Eng. J. Med. 329(7):459-465) and has been accepted for fast-track FDA approval under the trademark BEXXAR (*see, e.g.*, The Scientist 14[4]:16, February 21, 2000). FB1 was described by Nozawa *et al.*, 1999, Fukushima J. Med. Sci. 45:1-11.

Many of the anti-CD20 antibodies are available commercially, either as the purified monoclonal antibody or the antibody-secreting hybridoma. Sources of the anti-CD20 antibodies or hybridomas include Lab Vision Corporation, Fremont, CA (93-1B3 antibody); Bioprobe BV, the Netherlands (B9E9, 93-1B3 and 109-3C2 hybridomas); Serotec, United Kingdom (2H7, 7D1 and H147 antibodies); ID Labs, Ontario, Canada (2H7

antibody); Ancell, Bayport, Minnesota (2H7 antibody); the American Type Culture Collection, Manassas, Virginia (chimeric 2H7-expressing cell line C273; 1F5 hybridoma).

Further, the sequence of some of the anti-CD20 antibodies is known. MEM-97 has been partially sequenced (Dubel *et al.*, 1994, J. Immunol. Methods 175:89-95). A comparison of the variable regions of the light and heavy chains of the anti-CD20 antibodies C2B8, B9E9, and 1F5 is provided in Fig. 1 of Schultz *et al.*, 2000, Cancer Research 60:6663-6669.

The anti-CD20 antibodies of the invention are preferably monoclonal, and may be multispecific, human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, and CD20 binding fragments of any of the above. The term "antibody," as used herein, refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site that immunospecifically binds CD20. The immunoglobulin molecules of the invention can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule.

In certain embodiments of the invention, CD20- human antigen-binding antibody fragments can be used to deplete a patient's B-cells and include, but are not limited to, Fab, Fab' and $F(ab')_2$, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a V_L or V_H domain. Antigen-binding antibody fragments, including single-chain antibodies, may comprise the CD20-binding variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, CH3 and CL domains. Also included in the invention are antigen-binding fragments also comprising any combination of variable region(s) with a hinge region, CH1, CH2, CH3 and CL domains. Preferably, the variable regions are derived human, murine (*e.g.*, mouse and rat), donkey, sheep, rabbit, goat, guinea pig, camelid, horse, or chicken antibodies. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries, from human B-cells, or from animals transgenic for one or more human immunoglobulin, as described *infra* and, for example in U.S. Patent No.5,939,598 by Kucherlapati *et al.*

The anti-CD20 antibodies that may be used in the methods of the present invention may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for different epitopes of CD20 or may be specific for both CD20 as well as for a heterologous protein. *See*, *e.g.*, PCT publications WO

93/17715; WO 92/08802; WO 91/00360; WO 92/05793; Tutt, *et al.*, 1991, J. Immunol. 147:60-69; U.S. Patent Nos. 4,474,893; 4,714,681; 4,925,648; 5,573,920; 5,601,819; Kostelny *et al.*, 1992, J. Immunol. 148:1547-1553.

Antibodies of the present invention may be described or specified in terms of the particular variable regions or CDRs they comprise. In certain embodiments antibodies of the invention comprise one or more CDRs of the anti-CD20 antibodies 2B8, FB1, 1F5, 2H7, 93-1B3, 109-3C2, B1, B9E9, 7D1, H147, L26, L27, and MEM-97. In a preferred embodiment, those antibodies comprise human constant regions. In a most preferred embodiment, those antibodies comprise human constant and framework regions. Methods of generating such antibodies are described below.

Additionally, anti-CD20 antibodies for use in the methods of the present invention may also be described or specified in terms of their primary structures. Antibodies having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% and most preferably at least 98% identity (as calculated using methods known in the art and described herein in Section 5.1.1) to the variable regions of 2B8, FB1, 1F5, 2H7, 93-1B3, 109-3C2, B1, B9E9, 7D1, H147, L26, L27, and MEM-97 are also included in the present invention.

The present invention further encompasses the use of an anti-CD20 antibody that has amino acid substitutions relative to a native anti-CD20 antibody that resulting in improved affinity for CD20 relative to the native antibody. Such an antibody is optionally humanized. An exemplary method for identifying anti-CD20 antibodies with increased affinity is through systematic mutagenesis and screening, preferably reiterative screening, for antibodies with improved affinity to CD20, for example as described by Wu *et al.*, 1998, Proc. Natl. Acad. Sci. U.S.A. 95:6037-6042.

Anti-CD20 antibodies useful in the methods of the present invention may also be described or specified in terms of their binding affinity to CD20. Preferred binding affinities include those with a dissociation constant or Kd less than 5×10^{-2} M, 10^{-2} M, 5×10^{-3} M, 10^{-3} M, 5×10^{-4} M, 10^{-4} M, 5×10^{-5} M, 10^{-5} M, 5×10^{-6} M, 10^{-6} M, 5×10^{-7} M, 10^{-7} M, 5×10^{-8} M, 10^{-8} M, 5×10^{-9} M, 10^{-9} M, 5×10^{-10} M, 10^{-10} M, 5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, 10^{-12} M, $5 \times {}^{-13}$ M, 10^{-13} M, 5×10^{-14} M, 10^{-14} M, 5×10^{-15} M, or 10^{-15} M.

The anti-CD20 antibodies useful in the methods of the invention include derivatives that are modified, *i.e,* by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from binding to CD20 or from depleting B-cells. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, *e.g.*, by glycosylation, acetylation,

pegylation, phosphylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, *etc.* Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, *etc.* Additionally, the derivative may contain one or more non-classical amino acids.

The anti-CD20 antibodies useful in the methods of the present invention may be generated by any suitable method known in the art. Polyclonal antibodies to CD20 can be produced by various procedures well known in the art. For example, CD20 can be administered to various host animals including, but not limited to, rabbits, mice, rats, *etc.* to induce the production of sera containing polyclonal antibodies specific for the protein. Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. Such adjuvants are also well known in the art.

Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow *et al.*, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed., 1988); Hammerling, *et al.*, in: Monoclonal Antibodies and T-Cell Hybridomas 563-681 (Elsevier, N.Y., 1981) (said references incorporated by reference in their entireties). The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art. In a non-limiting example, mice can be immunized with CD20 or a cell expressing CD20 or a fragment or derivative thereof. Once an immune response is detected, *e.g.*, antibodies specific for CD20 are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well known techniques to any suitable myeloma cells, for example cells from cell line SP20 available from the ATCC. Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in

the art for cells that secrete antibodies capable of binding CD20. Ascites fluid, which generally contains high levels of antibodies, can be generated by injecting mice with positive hybridoma clones.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, Fab and $F(ab')_2$ fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments). $F(ab')_2$ fragments contain the variable region, the light chain constant region and the CH 1 domain of the heavy chain.

For example, the anti-CD20 antibodies useful in the methods of the present invention can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the nucleic acid sequences encoding them. In a particular embodiment, such phage can be utilized to display antigen binding domains expressed from a repertoire or combinatorial antibody library (*e.g.*, human or murine). In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the nucleic acid sequences encoding them. In particular, DNA sequences encoding V_H and V_L domains are amplified from animal cDNA libraries (*e.g.*, human or murine cDNA libraries of lymphoid tissues). The DNA encoding the V_H and V_L domains are recombined together with an scFv linker by PCR and cloned into a phagemid vector (*e.g.*, p CANTAB 6 or pComb 3 HSS). The vector is electroporated in *E. coli* and the *E. coli* is infected with helper phage. Phage used in these methods are typically filamentous phage including fd and Ml3 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Phage expressing an antigen binding domain that binds to CD20 can be selected or identified with antigen *e.g.*, using labeled antigen or antigen bound or captured to a solid surface or bead. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman *et al.*, 1995, J. Immunol. Methods 182:41-50; Ames *et al.*, 1995, J. Immunol. Methods 184:177-186; Kettleborough *et al.*, 1994, Eur. J. Immunol. 24:952-958; Persic *et al.*, 1997, Gene 187:9-18; Burton *et al.*, 1994, Advances in Immunology, 191-280; PCT Application No. PCT/GB91/O1 134; PCT Publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/1 1236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225;

5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, *e.g.*, as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and $F(ab')_2$ fragments can also be employed using methods known in the art such as those disclosed in PCT publication WO 92/22324; Mullinax *et al.*, BioTechniques 1992, 12(6):864-869; and Sawai *et al.*, 1995, AJRI 34:26-34; and Better *et al.*, 1988, Science 240:1041-1043 (said references incorporated by reference in their entireties).

Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patents 4,946,778 and 5,258,498; Huston *et al.*, 1991, Methods in Enzymology 203:46-88 ; Shu *et al.*, 1993, PNAS 90:7995-7999; and Skerra *et al.*, 1988, Science 240:1038-1040 . For some uses, including *in vivo* use of antibodies in humans and *in vitro* proliferation or cytotoxicity assays, it is preferable to use chimeric, humanized, or human antibodies. A chimeric antibody is a molecule in which different portions of the antibody are derived from different animal species, such as antibodies having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Methods for producing chimeric antibodies are known in the art. *See e.g.*, Morrison, Science, 1985, 229:1202 ; Oi *et al.*, 1986, BioTechniques 4:214; Gillies *et al.*, 1989, J. Immunol. Methods 125:191-202; U.S. Patent Nos. 5,807,715; 4,816,567; and 4,816,397, which are incorporated herein by reference in their entirety. Humanized antibodies are antibody molecules from non-human species antibody that binds the desired antigen having one or more CDRs from the non-human species and framework and constant regions from a human immunoglobulin molecule. Often, framework residues in the human framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen binding. These framework substitutions are identified by methods well known in the art, *e.g.*, by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (*See, e.g.*, Queen *et al.*, U.S. Patent No. 5,585,089; Riechmann *et al.*, , 1988, Nature 332:323 , which are incorporated herein by reference in their entireties.) Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 9

1/09967; U.S. Patent Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, Molecular Immunology, 1991, 28(4/5):489-498; Studnicka *et al.*, 1994, Protein Engineering 7(6):805-814; Roguska. *et al.*, 1994, PNAS 91:969-973), and chain shuffling (U.S. Patent No. 5,565,332).

Completely human antibodies are particularly desirable for therapeutic treatment of human subjects. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See also, U.S. Patent Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated herein by reference in its entirety.

Human antibodies can also be produced using transgenic mice which express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, *e.g.*, all or a portion of CD20. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, *see*, Lonberg and Huszar, 1995, Int. Rev. Immunol. 13:65-93. For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, *see, e.g.*, PCT publications WO 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Patent Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; 5,885,793; 5,916,771; and 5,939,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, CA), Genpharm (San Jose, CA) and Medarex (Princeton, NJ) can

be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, *e.g.*, a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope (Jespers *et al.*, 1994, Bio/technology 12:899-903).

Further, antibodies to CD20 can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" CD20 using techniques well known to those skilled in the art. (*See, e.g.*, Greenspan & Bona, 1989, FASEB J. 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438). Fab fragments of such anti-idiotypes can be used in therapeutic regimens to elicit an individual's own immune response against CD20 and B-cells.

The anti-CD20 antibodies useful in the methods of the invention may further be recombinantly fused to a heterologous protein at the N– or C-terminus or chemically conjugated (including covalently and non-covalently conjugations) to cytotoxic agents, proteins or other compositions.

5.1.1 DETERMINING SEQUENCE HOMOLOGY AMONG ANTI-CD20 ANTIBODIES

To determine the percent identity of two amino acid sequences or of two nucleic acids, *e.g.* between the sequences of the variable regions of two anti-CD20 antibodies, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = # of identical positions/total # of positions (*e.g.*, overlapping positions) x 100). In one embodiment, the two sequences are the same length.

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. USA 87:2264-2268, modified as in Karlin and Altschul, 1993, Proc. Natl. Acad. Sci. USA 90:5873-5877. Such an algorithm is

incorporated into the NBLAST and XBLAST programs of Altschul, *et al.*, 1990, J. Mol. Biol. 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid encoding a SCA-1 modifier protein. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a SCA-1 modifier protein. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, 1997, Nucleic Acids Res. 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (*Id.*). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. *See* http://www.ncbi.nlm.nih.gov. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. Additional algorithms for sequence analysis are known in the art and include ADVANCE and ADAM as described in Torellis and Robotti, 1994, Comput. Appl. Biosci. 10:3-5; and FASTA described in Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. 85:2444-8. Within FASTA, ktup is a control option that sets the sensitivity and speed of the search. If ktup=2, similar regions in the two sequences being compared are found by looking at pairs of aligned residues; if ktup=1, single aligned amino acids are examined. ktup can be set to 2 or 1 for protein sequences, or from 1 to 6 for DNA sequences. The default if ktup is not specified is 2 for proteins and 6 for DNA. For a further description of FASTA parameters, see http://bioweb.pasteur.fr/docs/man/man/fasta.1.html#sect2, the contents of which are incorporated herein by reference.

Alternatively, protein sequence alignment may be carried out using the CLUSTAL W algorithm, as described by Higgins *et al.*, 1996, Methods Enzymol. 266:383-402.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

5.2 BINDING ASSAYS

Methods of demonstrating the ability of an antibody to bind to CD20, and thus its usefulness in the disclosed methods, are described herein.

A putative anti-CD20 antibody may be assayed for immunospecific binding to CD20 by any method known in the art. The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as Western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art (*see, e.g.*, Ausubel *et. al.*, eds., 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference herein in its entirety). Exemplary immunoassays are described briefly below (but are not intended by way of limitation).

Immunoprecipitation protocols generally comprise lysing a population of cells in a lysis buffer such as RIPA buffer (1% NP-40 or Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 1% Trasylol) supplemented with protein phosphatase and/or protease inhibitors (*e.g.*, EDTA, PMSF, aprotinin, sodium vanadate), adding the antibody to the cell lysate, incubating for a period of time (*e.g.*, 1-4 hours) at 40° C, adding protein A and/or protein G sepharose beads to the cell lysate, incubating for about an hour or more at 40° C, washing the beads in lysis buffer and resuspending the beads in SDS/sample buffer. The ability of the antibody to immunoprecipitate CD20 can be assessed by, *e.g.*, Western blot analysis. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the binding of the antibody to CD20 and decrease the background (*e.g.*, pre-clearing the cell lysate with sepharose beads). For further discussion regarding immunoprecipitation protocols *see, e.g.*, Ausubel *et al., eds.*, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.16.1.

Western blot analysis generally comprises preparing protein samples, electrophoresis of the protein samples in a polyacrylamide gel (*e.g.*, 8%- 20% SDS-PAGE depending on the molecular weight of the antigen), transferring the protein sample from the polyacrylamide gel to a membrane such as nitrocellulose, PVDF or nylon, incubating the membrane in blocking solution (*e.g.*, PBS with 3% BSA or non-fat milk), washing the membrane in washing buffer (*e.g.*, PBS-Tween 20), blocking the membrane with primary antibody (*i.e.*, the putative anti-CD20 antibody) diluted in blocking buffer, washing the

membrane in washing buffer, incubating the membrane with a secondary antibody (which recognizes the primary antibody, *e.g.*, an anti-human antibody) conjugated to an enzyme substrate (*e.g.*, horseradish peroxidase or alkaline phosphatase) or radioactive molecule (*e.g.*, ^{32}P or ^{125}I) diluted in blocking buffer, washing the membrane in wash buffer, and detecting the presence of the secondary antibody. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected and to reduce the background noise. For further discussion regarding Western blot protocols *see, e.g.*, Ausubel *et al.*, eds., 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.8.1.

ELISAs comprise preparing antigen (*i.e.*, CD20), coating the well of a 96 well microtiter plate with the CD20, adding the antibody conjugated to a detectable compound such as an enzyme (*e.g.*, horseradish peroxidase or alkaline phosphatase) to the well and incubating for a period of time, and detecting the presence of the antibody. In ELISAs the antibody does not have to be conjugated to a detectable compound; instead, a second antibody (which recognizes the antibody of interest) conjugated to a detectable compound may be added to the well. Further, instead of coating the well with the antigen, the antibody may be coated to the well. In this case, a second antibody conjugated to a detectable compound may be added following the addition of CD20 protein to the coated well. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art. For further discussion regarding ELISAs *see, e.g.*, Ausubel *et al.*, eds., 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 11.2.1.

The binding affinity of an antibody to CD20 and the off-rate of an antibody CD20 interaction can be determined by competitive binding assays. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled CD20 (*e.g.*, ^{3}H or ^{125}I) with the antibody of interest in the presence of increasing amounts of unlabeled CD20, and the detection of the antibody bound to the labeled CD20. The affinity of the antibody for CD20 and the binding off-rates can then be determined from the data by Scatchard plot analysis. Competition with a second antibody can also be determined using radioimmunoassays. In this case, CD20 is incubated with the antibody of interest conjugated to a labeled compound (*e.g.*, ^{3}H or ^{125}I) in the presence of increasing amounts of an unlabeled second antibody.

5.3 METHODS OF PRODUCING ANTI-CD20 ANTIBODIES

The proteins, including antibodies, of the invention can be produced by any method known in the art for the synthesis of proteins, in particular, by chemical synthesis or preferably, by recombinant expression techniques.

Recombinant expression of an anti-CD20 antibody, including a fragment, derivative or analog thereof, *e.g.*, a heavy or light chain of an anti-CD20 antibody) requires construction of an expression vector containing a nucleic acid that encodes the anti-CD20 antibody. Once a nucleic acid encoding an anti-CD20 antibody has been obtained, the vector for the production of the anti-CD20 antibody may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing an anti-CD20 antibody by expressing a nucleic acid containing a nucleotide sequence encoding said anti-CD20 antibody are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. The invention, thus, provides replicable vectors comprising a nucleotide sequence encoding an anti-CD20 antibody operably linked to a promoter. The anti-CD20 antibody nucleotide sequence may encode a heavy or light chain thereof, or a heavy or light chain variable domain, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the anti-CD20 antibody molecule (*see*, *e.g.*, PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Patent No. 5,122,464) and the variable domain of the anti-CD20 antibody may be cloned into such a vector for expression of the entire heavy or light chain.

The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce a protein of the invention. Thus, the invention encompasses host cells containing a nucleic acid encoding a protein of the invention, operably linked to a heterologous promoter. In preferred embodiments for the expression of double-chained antibodies, vectors encoding both the heavy and light chains may be co-expressed in the host cell for expression of the entire immunoglobulin molecule, as detailed below.

A variety of host-expression vector systems may be utilized to express the protein molecules of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express a protein of the invention *in situ*. These include but are not

limited to microorganisms such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing antibody coding sequences; yeast (*e.g.*, *Saccharomyces*, Pichia) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing antibody coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing antibody coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter). Preferably, bacterial cells such as *Escherichia coli*, and more preferably, eukaryotic cells, especially for the expression of whole recombinant antibody molecules, are used for the expression of a recombinant protein of the invention. For example, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for proteins of the invention (Foecking *et al.*, 1986, Gene 45:101; Cockett *et al.*, 1990, Bio/Technology 8:2).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the folding and post-translation modification requirements protein being expressed. Where possible, when a large quantity of an anti-CD20 antibody is to be produced, for the generation of pharmaceutical compositions comprising the anti-CD20 antibody or fragment or derivative thereof, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther *et al.*, 1983, EMBO 1. 2:1791), in which the anti-CD20 antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 24:5503-5509); and the like. pGEX vectors may also be used to express fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to matrix glutathioneagarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include

thrombin or factor Xa protease cleavage sites so that the cloned anti-CD20 antibody can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The anti-CD20 antibody coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the coding sequence of the anti-CD20 antibody may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non- essential region of the viral genome (*e.g.*, region El or E3) will result in a recombinant virus that is viable and capable of expressing the anti-CD20 antibody in infected hosts. (*See, e.g.*, Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 8 1:355-359). Specific initiation signals may also be required for efficient translation of inserted coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, *etc.* (*see*, Bittner *et al.*, 1987, Methods in Enzymol. 153:51-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of anti-CD20 antibodies may be important for the binding and/or activities of the antibodies. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the anti-CD20 antibody expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, Hela, COS, MDCK, 293, 3T3, and W138.

For long-term, high-yield production of recombinant anti-CD20 antibodies, stable expression is preferred. For example, cell lines which stably express an anti-CD20 antibody may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express an anti-CD20 antibody for use in the methods of the present invention.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1992, Proc. Natl. Acad. Sci. USA 48:202), and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, Cell 22:8-17) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler *et al.*, 1980, Proc. Natl. Acad. Sci. USA 77:357; O'Hare *et al.*, 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95 ; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932 ; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62: 191-217; May, 1993, TIB TECH 11(5):155-215); and hygro, which confers resistance to hygromycin (Santerre *et al.*, 1984, Gene 30:147). Methods commonly known in the art of recombinant DNA technology may be routinely applied to select the desired recombinant clone, and such methods are described, for example, in Ausubel *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990); and in Chapters 12 and 13, Dracopoli *et al.* (eds), Current Protocols in Human Genetics, John Wiley & Sons, NY (1994); Colberre-Garapin *et al.*, 1981, J. Mol. Biol. 150:1, which are incorporated by reference herein in their entireties.

The expression levels of an anti-CD20 antibody can be increased by vector amplification (for a review, see Bebbington and Hentschel, "The Use of Vectors Based on

Gene Amplification for the Expression of Cloned Genes in Mammalian Cells in DNY Cloning", Vol.3. (Academic Press, New York, 1987)). When a marker in the vector system expressing the anti-CD20 antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the antibody gene, production of the anti-CD20 antibody will also increase (Crouse *et al.*, 1983, Mol. Cell. Biol. 3:257).

In certain specific embodiments, the host cell may be co-transfected with two expression vectors encoding an anti-CD20 antibody, the first vector encoding a heavy chain derived protein and the second vector encoding a light chain derived protein. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain proteins. Alternatively, a single vector may be used which encodes, and is capable of expressing, both heavy and light chain proteins. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, 1986, Nature 322:52 (1986); Kohler, 1980, Proc. Natl. Acad. Sci. USA 77:2 197). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

Once an anti-CD20 antibody has been produced by an animal, chemically synthesized, or recombinantly expressed, it may be purified by any method known in the art for purification of proteins, for example, by chromatography (*e.g.*, ion exchange; affinity, particularly by affinity for the specific antigen (*i.e.*, CD20); Protein A; or affinity for a heterologous fusion partner wherein the protein is a fusion protein; and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

The present invention encompasses the use of anti-CD20 antibodies recombinantly fused or chemically conjugated (including both covalent and non-covalent conjugation) to heterologous proteins (of preferably at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or at least 100 amino acids) to generate fusion proteins. The fusion does not necessarily need to be direct, but may occur through linker sequences.

5.4 TOXINS

As discussed *supra*, the present invention provides methods for reduction or prevention of an immune response to an immunogenic molecule in a patient by administering to the patient an anti-CD20 monoclonal antibody in an amount sufficient to deplete the patient's circulating B-cells.

In certain embodiments of the invention, the immunogenic molecule is a toxin, *i.e.*, a cytostatic or cytocidal agent. The toxin is preferably covalently linked to a second protein in which the toxin's fusion partner targets the toxin to a particular cell type. The covalent linkage can be by way of conjugation or, more preferably, by way of recombinant expression as a fusion protein in which the toxin is translated in frame at the – or C-terminus of the second protein.

Chimeric toxins, recombinant toxins, and immunotoxins (as will be described below, such molecules include antibody-toxin conjugates or recombinant fusion proteins comprising toxin moieties) are a relatively new group of macromolecules that are being developed for use in a variety of human illnesses. The underlying therapeutic principle is the joining of a toxin molecule to a targeting molecule of high specificity. The targeting molecule then delivers the toxin to the unwanted cell or tissue, where the toxin portion of the molecule is internalized and poisons the cell. Chimeric toxins, recombinant toxins, and immunotoxins are potentially useful in the treatment of cancer (both solid tumors and hematological malignancies), autoimmune diseases (*e.g.*, rheumatoid arthritis, diabetes mellitus type 1 and multiple sclerosis), and other conditions such as Acquired Immunodeficiency Syndrome (AIDS), graft versus host disease (GVHD), vascular restenosis, and rejection of organ transplants (Vitetta, *et al.* 1993, Immunology Today 14:252-259; Biro *et al.*, 1992, Circ. Res. 71:640-5; and Wawrzynczak and Derbyshire, 1992, Immunology Today 13:381-383). Targeting molecules utilized to convey various toxins include monoclonal antibodies and antibody fragments, growth factors (*e.g.*, epidermal growth factor), cytokines (*e.g.*, interleukin-2), and plant lectins. The principal toxins (or their fragments) used currently are bacterial or plant in origin and include, but are not limited to, ricin, *Diphtheria* toxin and *Pseudomonas* exotoxin A (Vitetta *et al.*, Immunology Today 14: 252-259; and Wawrzynczak and Derbyshire, 1992, Immunology Today 13: 381-383). Other plant and fungal toxins (*e.g.*, gelonin, saporin, bryodin) known as ribosome-inactivating proteins have had their genes cloned in preparation for possible use as immunotoxins. Ribosome-inactivating proteins are advantageous because many of them are single-chain, low molecular weight proteins (Wawrzynczak, 1991, Br. J. Cancer 64: 624-630; Francisco *et al.*, 1997, J. Biol. Chem. 39:24165-24169).

A general method of preparation of toxin conjugates may involve use of thiol-crosslinking reagents such as the heterobifunctional cross-linking reagent N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), which attack the primary amino groups on the second protein and by disulfide exchange, attach the toxin to the second protein (*e.g.*, a targeting protein such as an antibody or growth factor).

In one embodiment, the toxin is in the form of an immunotoxin comprising an antibody with specificity for a tumor- or disease-cell related cell surface receptor or antigen. In a preferred mode of the embodiment, the immunotoxin is a single-chain immunotoxin ("SCIT"), which comprises the Fv portion of an internalizing mAb linked to a truncated toxin that works inside the cell to stop protein synthesis. In another embodiment, the toxin is in the form of a fusion protein in which the toxin is fused to a ligand such as a growth factor or other signaling molecule, *e.g.*, a cytokine, that targets the toxin to tumor or disease cells that express a receptor for the ligand molecule.

Toxins suitable for use as immunogenic molecules according to the methods of the invention include molecules comprising plant-, fungus-, or bacteria-derived toxins, such as, for example, abrin, ricin A, *Pseudomonas* exotoxin, *Diphtheria* toxin, *Clostridium baratii* type F-like toxin, *Clostridium butyricum* type E-like toxin, *Staphylococcus* enterotoxin A, botulinum toxin, tetanus toxin, modeccin, bungarotoxin, shigatoxin, cholera toxin, bryodin, saporin or gelonin.

In one embodiment of the invention, the immunogenic molecule comprises *Pseudomonas* exotoxin. In a preferred mode of the embodiment, the *Pseudomonas* exotoxin is used as a genetically modified form termed PE40, which is a truncated form of the toxin in which the cell binding domain is deleted (*see*, Kondo *et al.*, 1988, J. Biol. Chem. 263:9470-9475). In another preferred mode of the embodiment, the *Pseudomonas* exotoxin is PE38, another truncated form of the toxin in which the cell binding domain is deleted (*see,* Brinkmann *et al.*, 1991, Proc Natl Acad Sci USA 88:8616-20). Because these forms of the exotoxin lacks a cell binding domain, they have to be administered covalently linked to an internalizing molecule such as a an internalizing monoclonal antibody.

When the immunogenic molecule comprises ricin A, the molecule can be glycosylated (for example by expression in a eukaryotic cell) or deglycosylated (for example by expression in a bacterial cell). In a preferred embodiment, the immunogenic molecule is deglycosylated ricin A due to its potency and lengthy half-life.

5.4.1 IMMUNOTOXINS

In certain embodiments of the invention, the immunogenic molecule is a monoclonal antibody-targeted toxin, more preferably a SCIT. The following embodiments are merely exemplary and the methods of the present invention encompass any immunotoxin whose administration to a patient elicits a humoral immune response.

In one embodiment, the immunotoxin is BR96 sFv-PE40, a single chain immunotoxin whose antibody portion recognizes the Le^y antigen present on a number of

carcinomas and whose toxin portion is a 40 kD form of *Pseudomonas* toxin lacking its cell-binding domain (PE40).

In another embodiment, the immunotoxin is K305Fab-SEA(D227A), an immunotoxin comprising the Fab portion of K305, an antibody against the high-molecular-weight melanoma-associated antigen, and a mutant form of the superantigen staphylococcal enterotoxin A (Tordsson *et al.*, 2000, Cancer Immunol. Immunother. 48:691-702).

In another embodiment, the immunotoxin is C242Fab-SEA, an immunotoxin comprising the Fab portion of C242, a monoclonal antibody immunoreactive with human colon carcinomas, and the superantigen staphylococcal enterotoxin A (Dohlsten *et al.*, 1995, Cancer Immunol. Immunother. 41:162-68).

In yet another embodiment, the immunotoxin comprises an antibody selected from MOC31 (an antibody against epithelial glycoprotein 2), BM7 (an antibody against MUC-1 mucin) and 425.3 (an antibody against the epidermal growth factor receptor), all of which are immunoreactive with human breast cancer cells, conjugated to *Pseudomonas* exotoxin (Engebraaten *et al.*, 2000, Int. J. Cancer 88:970-76).

In yet another embodiment, the immunotoxin is LMB-2, which comprises an antibody to the IL-2 receptor alpha subunit fused to PE38 (Onda *et al.*, 2000, J. Immunol. 165:7150-56).

In yet another embodiment, the immunotoxin is K1-LysPE38QQR, which is a chemical conjugate between K1, a monoclonal antibody which recognizes mesothelin, a cell surface antigen that is overexpressed in epithelial ovarian cancer cells and in malignant mesotheliomas, and a form of PE38 with altered chemical conjugation properties (Hassan *et al.*, 2000, J. Immunother. 23:473-79).

In yet another embodiment, the immunotoxin is RFB4 (dsFv)-PE38(BL22), which comprises an Fv fragment of the anti-CD22 antibody RFB4, fused to PE38 (Kreitman *et al.*, 2000, Clin. Cancer Res. 6(4):1476-87). RFB4 (dsFv)-PE38 recognizes CD22-expressing cells, including B cell malignancies.

In yet other embodiments, the immunotoxin is MR1(Fv)-PE38 (Beers *et al.*, 2000, Clin. Canc. Res. 6:2835-43); DT390-scFvUCHT1 (an anti T-cell immunotoxin comprising a Diphtheria toxin fragment and an anti-CD30 antibody; Liu *et al.*, 2000, Protein Expr. Purif. 19(2):304-11); Ki-4(scFv)-ETA' (an immunotoxin with activity against Hodgkin's disease cells comprising a *Pseudomonas* exotoxin fragment and an anti-CD30 antibody; Barth *et al.*, 2000, Blood 95:3909-14); RFT5(scFv)-ETA' (an immunotoxin with activity against Hodgkin's disease cells comprising an anti-CD25 antibody and a *Pseudomonas* exotoxin fragment; Barth *et al.*, 2000, Int. J. Cancer 86:718-24); or

RFT5.dgA (an immunotoxin with activity against Hodgkin's disease cells comprising an anti-CD25 antibody and deglycosylated Ricin A chain; Schnell *et al.*, 2000, Leukemia 14:129-35).

5.4.2 OTHER TOXIN-CONTAINING FUSION PROTEINS

In certain embodiments of the invention, the toxin to which prevention of an immune response is desired is a non-monoclonal antibody targeted toxin. Exemplary non-monoclonal antibody targeted toxins are provided below.

In certain specific embodiments, the non-monoclonal antibody targeted toxin is denileukin diftitox, which is sold by Ligand Pharmaceuticals, Inc. under the trademark ONTAK®. Denileukin diftitox is a single chain fusion protein composed of IL-2 and a truncated form of the bacterial protein toxin from *Diphtheria*. More specifically, denileukin diftitox is a recombinant DNA-derived cytotoxic protein composed of the amino acid sequences for *Diphtheria* toxin fragments A and B (Met 1-Thr 387)-His followed by the sequences for interleukin-2 (IL-2; Ala 1-Thr 133), and is produced in an *E. coli* expression system. The fusion protein designed to direct the cytocidal action of *Diphtheria* toxin to cells which express the IL-2 receptor. The human IL-2 receptor exists in three forms, low (CD25), intermediate (CD122/CD132) and high (CD25/CD122/CD132) affinity. The high affinity form of the IL-2 receptor is usually found only on activated T-cells, activated B-cells and activated macrophages. Malignant cells expressing one or more of the subunits of the IL-2 receptor are found in certain leukemias and lymphomas including cutaneous T-cell lymphoma. *Ex vivo* studies suggest that denileukin diftitox interacts with the high affinity IL-2 receptor on the cell surface and inhibits cellular protein synthesis, resulting in cell death within hours. The fusion protein has been approved by the FDA for the treatment of patients with persistent or recurrent cutaneous T-cell lymphoma (CTCL) whose malignant cells express the CD25 component of the interleukin-2 (IL-2) receptor.

Other non-monoclonal antibody targeted toxins are known to those of skill in the art and can be used in accordance with the disclosed methods. Exemplary non-monoclonal antibody targeted toxins include IL13-PE38QQR (a cytotoxin against interleukin-13 receptor-expressing cells, such as Kaposi's sarcoma cells, comprising interleukin-13 and a *Pseudomonas* exotoxin fragment; Husain and Puri, 2000, Blood 95:3506-13); GnRH-PAP (a cytotoxin against gynecologic tumors comprising GnRH (gonadotrophin releasing hormone) and PAP, pokeweed antiviral protein, a ribosome inactivating protein); ATF-PE38 (comprising a urokinase fragment and PE38, with specificity to cells that express the urokinase plasminogen activator receptor; Rajagopal and

Kreitman, 2000, J. Biol. Chem. 275:7566-73); and CD30L-ETA' (comprising CD30 ligand and a fragment of Pseudomonas exotoxin; the CD30 ligand directs the toxin to the CD30-expressing Hodgkin's disease cells; Barth *et al.*, 1999, Cytokines Cell Mol. Ther. 5:69-78).

5.5 ENZYMES

The present invention provides methods for reduction or prevention of an immune response to, *inter alia*, an enzyme in a patient by administering to the patient an anti-CD20 monoclonal antibody in an amount sufficient to deplete the patient's circulating B-cells prior to, concurrently with or after the administration of the enzyme to the patient. The enzyme is preferably a pro-drug converting enzyme (as described in Section 5.5.1 *infra*) or another enzyme administered for therapeutic purposes. For example, the enzyme can be a DNAse I with DNA degrading properties, targeted to a tumor cell (for example by means of an antibody against a tumor cell antigen), and whose delivery to a tumor cell is cytotoxic to the tumor cell (*see*, *e.g.*, Linardou *et al.*, 2000, Int. J. Cancer 86:561-69).

5.5.1 PRO-DRUG CONVERTING ENZYMES

As discussed above, in certain embodiments of the invention, the immunogenic molecule comprises a pro-drug converting enzyme. Drugs that normally have considerable toxicity to normal tissues can be administered as inactive pro-drugs and converted to their cytotoxic form by the enzyme that has been localized to the tumor microenvironment by a targeting molecule such as an antibody. This approach results in enzymatic conversion of the inactive pro-drug to the active drug specifically in tumor tissue, thus reducing exposure of normal tissue to the drug while maximizing concentrations in tumor tissue.

The pro-drug converting enzyme can be expressed as a fusion protein with or conjugated to an antibody to target the pro-drug converting enzyme to a specific cell type. The pro-drug converting enzyme then cleaves nontoxic pro-drugs and release chemotherapeutically active drugs at a tumor site to which it has been targeted by the antibody. Monoclonal antibodies that remain at the cell surface are preferred fusion partners for pro-drug converting enzymes. Alternatively, the pro-drug converting enzyme can be expressed as a fusion protein with or conjugated to a growth factor or other signaling molecule, such as a cytokine, to target the enzyme to cells that express a receptor for the growth factor or signaling molecule.

In embodiments of the invention in which the immunogenic molecule comprises a pro-drug converting enzyme, the immunogenic molecule is administered prior

to the pro-drug, which is then activated at the site to which the pro-drug converting enzyme has been targeted. Regimens of administration of pro-drug converting enzymes and pro-drugs are known to those of skill in the art.

Exemplary pro-drug converting enzymes are carboxypeptidase G2, beta-glucuronidase, penicillin-V-amidase, penicillin-G-amidase, β-lactamase, β-glucosidase, nitroreductase and carboxypeptidase A.

In an exemplary embodiment, the immunogenic molecule comprises the cloned binding region (sFv) of the L49 monoclonal antibody that binds to the p97 antigen (a human melanoma-associated antigen) and the highly specific enzyme β-lactamase. The p97 antigen is also expressed in many ovarian, breast and lung cancers. When the L49 monoclonal antibody binds to its antigen, the mAb does not penetrate inside the cell, rather it sits on the cell surface. In the form of an sFv-β-lactamase fusion protein, L49 is retained on the cell surface leaving the fused enzymatic portion exposed and available to activate pro-drug substrates. In a preferred mode of the embodiment, the L49 sFv-β-lactamase fusion protein is administered with a cephalosporin derivative of the clinically approved drug melphalan, an alkylating agent. (Kerr *et al.*, 1999, Bioconj. Chem. 10:1084-89). When the cephalosporin group is cleaved from the melphalan unit by the catalytic action of β-lactamase, the fully active agent melphalan is generated.

In another exemplary embodiment, the immunogenic molecule is a fusion between the single-chain anti-carcinoma antibody 323/A3 and beta-glucuronidase (Haisma *et al.*, 1998, Cancer Immunol Immunother 45(5):266-72), which catalyzes the conversion of the pro-drug N-[4-doxorubicin-N-carbonyl(oxymethyl)phenyl]-O-beta-glucuronyl carbamate to the cytotoxic drug doxorubicin.

5.6 GENE THERAPY

In certain specific embodiments of the invention, an anti-CD20 antibody is administered to deplete B-cells in order to prevent an immune response to a protein expressed by a gene therapy vector. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleic acid. The nucleic acid is often a replicable viral genome engineered to express a heterologous protein that mediates a therapeutic effect. Gene therapy is often limited by the patient's immune response to viral vectors of gene therapy, such as adenovirus vectors (*see, e.g.*, Yang *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:4407-4411). For example, in an animal trial, administration of an adenovirus harboring a factor IX (for treatment of hemophilia) to normal mice results in only short term expression of the factor IX gene and re-administration of the vector is

prevented because of neutralizing antibodies to the adenovirus. In contrast, administration of the same gene therapy vector to *nude* mice results in long term expression of the factor IX (Dai *et al.*, 1995, Proc. Natl. Acad. Sci U.S.A. 92:1401-1405). Thus, suppression of the immune response to the adenovirus vector would be expected to prolong the usefulness of gene therapy. Immune responses against both the native proteins of the gene therapy vector and the heterologous, therapeutic protein expressed by the vector can both be prevented by the disclosed methods.

The anti-CD20 antibody can be used in conjunction with any gene therapy vector available in the art. Exemplary vectors and methods are described below.

For general reviews of the methods of gene therapy, *see*, Goldspiel *et al.*, 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH 1, l(5):155-215. Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); and Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990).

Delivery of a gene therapy vector into a subject may be either direct, in which case the subject is directly exposed to the nucleic acid or nucleic acid- carrying vector, or indirect, in which case, cells are first transformed with the nucleic acids *in vitro*, then transplanted into the subject. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid sequences are directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, for example by constructing them as part of an appropriate nucleic acid expression vector and administering the vector so that the nucleic acid sequences become intracellular. Gene therapy vectors can be administered by infection using defective or attenuated retrovirals or other viral vectors (*see*, *e.g.*, U.S. Patent No. 4,980,286); direct injection of naked DNA; use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont); coating with lipids or cell-surface receptors or transfecting agents; encapsulation in liposomes, microparticles, or microcapsules; administration in linkage to a peptide which is known to enter the nucleus; administration in linkage to a ligand subject to receptor-mediated endocytosis (*see*, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432) (which can be used to target cell types specifically expressing the receptors); *etc.* In another embodiment, nucleic acid-ligand

complexes can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (*see, e.g.*, PCT Publications WO 92/06 180; WO 92/22635; W092/20316; W093/14188, and WO 93/20221). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra *et al.*, 1989, Nature 342:435-438).

In a specific embodiment, viral vectors that contain nucleic acid sequences encoding an antibody of the invention are used. For example, a retroviral vector can be used (see Miller *et al.*, 1993, Meth. Enzymol. 217:581-599). These retroviral vectors contain the components necessary for the correct packaging of the viral genome and integration into the host cell DNA. The nucleic acid sequences encoding the antibody to be used in gene therapy are cloned into one or more vectors, thereby facilitating delivery of the gene into a subject. More detail about retroviral vectors can be found in Boesen *et al.*, 1994, Biotherapy 6:29 1-302, which describes the use of a retroviral vector to deliver the mdr 1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes *et al.*, 1994, J. Clin. Invest. 93:644-651; Klein *et al.*, 1994, Blood 83:1467-1473; Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114.

Another approach to gene therapy involves transferring a gene encoding a therapeutic protein to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a subject.

In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell mediated gene transfer, spheroplast fusion, *etc.* Numerous techniques are known in the art for the introduction of foreign genes into cells (*see, e.g.*, Loeffler and Behr, 1993, Meth. Enzymol. 217:599-618; Cohen *et al.*, 1993, Meth. Enzymol. 217:618-644; Cline, 1985,

Pharmac. Ther. 29:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a subject by various methods known in the art. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, subject state, *etc.*, and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to fibroblasts; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, *etc.*

In a preferred embodiment, the cell used for gene therapy is autologous to the subject.

In an embodiment in which recombinant cells are used in gene therapy, nucleic acid sequences encoding an antibody are introduced into the cells such that they are expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used in accordance with this embodiment of the present invention (see *e.g.* PCT Publication WO 94/08598; Stemple and Anderson, 1992, Cell 71:973-985; Rheinwald, 1980, Meth. Cell Bio. 21A:229; and Pittelkow and Scott, 1986, Mayo Clinic Proc. 61:771).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

5.7 OTHER THERAPEUTIC MOLECULES

As discussed in Section 3 *supra*, the methods of the present invention can be used to reduce or prevent a humoral immune response to any therapeutic molecule that is immunogenic, provided that the therapeutic molecule is not a component of a tissue

transplant. Additional non-limiting examples of therapeutic molecules that have immunogenic properties and with which the methods of the present invention can be practiced include recombinant GM-CSF (Wadhwa *et al.*, 1999, Clin. Cancer Res. 5:1353-61) and recombinant thrombopoietin "TPO" (Wendling and Vainchenker, 1999, Hématologie 5:289-94).

5.8 THERAPEUTIC/PROPHYLACTIC ADMINISTRATION AND COMPOSITIONS

The invention provides methods of preventing a humoral immune response to an immunogenic molecule having therapeutic value in a subject by administering to the subject an amount of an anti-CD20 antibody effective to deplete the subject's peripheral B-cells. According to the present invention, prevention of a humoral immune response entails depletion of the patient's B-cells with an anti-CD20 antibody, which prolongs the half life of the immunogenic molecule in the patient's circulation and/or prevents neutralization of the immunogenic molecule, thereby increasing the therapeutic efficacy of the immunogenic molecule.

In a preferred aspect, the anti-CD20 antibody is substantially purified (*e.g.*, substantially free from substances that limit its effect or produce undesired side-effects). The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, *etc.*, and is preferably a mammal, and most preferably human.

Formulations and methods of administration that can be employed are described below; additional appropriate formulations and routes of administration can be selected from among those described herein below.

Various delivery systems are known and can be used to administer an anti-CD20 antibody in accordance with the methods of the present invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (*see, e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432). Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The anti-CD20 antibodies may be also administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and may be administered together with other biologically active agents, including but not limited to an immunogenic molecule. Administration can be systemic or local.

In a specific embodiment, it may be desirable to administer the anti-CD20 antibody by injection, by means of a catheter, by means of a suppository, or by means of an

implant, said implant being of a porous, non-porous, or gelatinous material, including a membrane, such as a sialastic membrane, or a fiber. Preferably, when administering an anti-CD20 antibody, care must be taken to use materials to which the anti-CD20 antibody does not absorb.

In another embodiment, the anti-CD20 antibody can be delivered in a vesicle, in particular a liposome (*see* Langer, 1990, Science 249:1527-1533; Treat *et al.*, 1989, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353- 365; Lopez-Berestein, ibid., pp. 317-327; see generally, *ibid.*)

In yet another embodiment, the anti-CD20 antibody can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, 1989, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald *et al.*, 1980, Surgery 88:507; Saudek *et al.*, 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, 1974, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida; Controlled Drug Bioavailability, Drug Product Design and Performance, 1984, Smolen and Ball (eds.), Wiley, New York; Ranger and Peppas, 1983, Macromol. Sci. Rev. Macromol. Chem. 23:61; see also Levy *et al.*, 1985, Science 228:190; During *et al.*, 1989, Ann. Neurol. 25:351; Howard *et al.*, 1989, J. Neurosurg. 71:105).

Other controlled release systems are discussed in the review by Langer, 1990, Science 249:1527-1533.

Pharmaceutical compositions comprising an amount of anti-CD20 antibody effective to deplete a patient's B-cells further comprise a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the anti-CD20 antibody is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol,

water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate) lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicles before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the anti-CD20 antibody, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the anti-CD20 antibodies are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered

amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The anti-CD20 antibodies may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multidose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The anti-CD20 antibodies may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the anti-CD20 antibodies may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the proteins may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

In a preferred embodiment, the pharmaceutical composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the pharmaceutical composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampule or sachette indicating the quantity of active agent, *i.e.*, the anti-CD20 antibody. Where the pharmaceutical of the invention is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the pharmaceutical composition comprising anti-CD20 antibody is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The anti-CD20 antibody compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration preferably for administration to a human.

Generally, the method of administration of the immunogenic molecule will depend on the type of immunogenic molecule being administered. Methods of administering immunogenic molecules that are components of gene therapy vectors are described in Section 5.6, *supra*. For immunogenic molecules that are administered as proteins, such as, but not limited to, immunotoxins or fusion proteins comprising antigenic proteins such as pro-drug converting enzymes, compositions and methods of administration are generally similar to those described for the anti-CD20 antibodies and will be readily apparent to those of skill in the art.

5.9 EFFECTIVE DOSE

The amount of the anti-CD20 antibody which will be effective to deplete a patient's B-cells in accordance with the methods of the present invention can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the timing of administration of the anti-CD20 antibody relative to administration of the immunogenic molecule, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from animal model test systems.

In one embodiment, the amount of anti-CD20 antibody administered is sufficient to deplete the patient's circulating B-cells by at least 25%, more preferably by at least 35%, yet more preferably by at least 50%, and most preferably by at least 60%. In other embodiments, the amount of anti-CD20 antibody administered is sufficient to deplete the patient's circulating B-cells by at least 75%, at least 85%, or at least 90%.

Toxicity and therapeutic efficacy of a particular anti-CD20 antibody in conjunction with a given immunogenic molecule can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of particular combinations of immunogenic molecules and anti-CD20 antibodies lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any immunogenic molecule administered in conjunction with an anti-CD20 antibody, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (*i.e.*, the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

Generally, the dosage of an anti-CD20 antibody administered to deplete a patient's B-cells is typically 0.1 mg/kg to 100 mg/kg of the patient's body weight. Preferably, the dosage administered to a patient is between 0.1 mg/kg and 20 mg/kg of the patient's body weight, more preferably 1 mg/kg to 10 mg/kg of the patient's body weight. In other embodiments, the dosage of the anti-CD20 antibody is 50 mg/m^2 to 1000 mg/m^2, more preferably 100 mg/m^2 to 750 mg/m^2, more preferably 200 mg/m^2 to 500 mg/m^2, and yet more preferably 300 mg/m^2 to 400 mg/m^2 of a patient's body surface area. Generally, the more 'human' an anti-CD20 antibody is, the longer its half-life within the human body, and the lower the dosages that be required to deplete the patient's B-cells.

5.10 MEASURING B CELL DEPLETION

Generally, in order to determine whether a patient's B-cells have been depleted, for example to identify suitable dosages of the anti-CD20 antibody and/or to measure the efficacy of treatment, B-cells in a patient's sample can be detected and counted by measuring the presence of surface transmembrane immunoglobulin. Anti-immunoglobulin antisera against different classes of immunoglobulins can be labeled with a fluorescent label and the number of B-cells in the sample measured by contacting the sample with the mixture of fluorescently labeled antisera and the cells with surface fluorescence counted in the sample.

Alternatively, B-cells can be quantified to determine the extent of their depletion by flow cytometry. In one embodiment, peripheral blood mononuclear cells (PBMC) are isolated by centrifugation. Antibodies against B cell markers (such as CD20, CD19 and/or CD33) which are conjugated to fluorochrome are added to allow the binding

of the antibodies to B cell specific surface markers. The cell mixture is then washed by one or more centrifugation and resuspension steps. The resulting cell population is analyzed by flow cytometry to determine the number of B-cells present in the sample, for example on an EPICS C (Coulter Electronics, Hileah, Fla.).

Other methods of measuring B cell depletion are known to those of skill in the art and can be used to determine the efficacy of an anti-CD20 antibody in B-cell depletion.

5.11 THERAPEUTIC REGIMENS

According to the present invention, an anti-CD20 antibody is administered to a patient in order to deplete a patient's B-cells so that a humoral immune response is not mounted against an immunogenic molecule that is administered to the patient for therapy.

In a highly preferred embodiment, the anti-CD20 antibody is administered prior to administration of the immunogenic molecule, so that the patient's B-cells are depleted prior to administration of the immunogenic molecule, but not so long prior to administration of the immunogenic molecule that the patient's B-cells regenerate prior to the administration of the immunogenic molecule. In specific modes of the embodiment, the immunogenic molecule is administered within about five hours, within about 12 hours, within about one day, within about two days, within about three days, within about five days, within about a week, within about ten days, within about two weeks or within about three weeks of administration of the anti-CD20 antibody.

In another embodiment of the invention, the anti-CD20 antibody is administered concurrently with administration of the immunogenic molecule. In yet another embodiment of the invention, the anti-CD20 antibody is administered following administration of the immunogenic molecule. In a preferred mode of the embodiment, the anti-CD20 antibody is administered no later than one week after the administration of the immunogenic molecule, more preferably no later than 3 days after the administration of the immunogenic molecule, more preferably no later than 1 day after the administration of the immunogenic molecule, and most preferably no later than 12 hours after the administration of the immunogenic molecule.

In certain embodiments of the invention, a course of anti-CD20 antibody comprising more than one dose of the antibody is administered to a patient. In one embodiment, the course is initiated and completed prior to administration of an immunogenic molecule to the patient. In another embodiment, the course is initiated prior to the first administration of the immunogenic molecule to the patient and completed

concurrently with the first administration of the immunogenic molecule to the patient. In yet another embodiment, the course is initiated prior to the first administration of the immunogenic molecule to the patient and completed (*i.e.*, the last dose is administered) concurrently with the first administration of the immunogenic molecule to the patient. In yet another embodiment, the course is initiated prior to the first administration of the immunogenic molecule to the patient and completed (*i.e.*, the last dose is administered) concurrently with the administration of the immunogenic molecule to the patient. In yet other embodiments, the course is initiated concurrently or following the first administration of the immunogenic molecule to the patient and completed after the first administration of the immunogenic molecule to the patient.

In one embodiment, a course of anti-CD20 antibody comprises two administrations of the antibody. In another embodiment, a course of anti-CD20 antibody comprises three administrations of the antibody. In yet other embodiments, a course of anti-CD20 antibody comprises four, five or six administrations of the antibody. The administrations can be separated by an average of about a week, about five days, about four days, about three days or about two days.

5.12 KITS

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with an anti-CD20 antibody and an immunogenic molecule, and optionally one or more pharmaceutical carriers. In one embodiment, the immunogenic molecule is BR96 sFv-PE40. In another embodiment, the immunogenic molecule is denileukin diftitox (ONTAK®).

Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

The invention is further described in the following examples which are in no way intended to limit the scope of the invention.

6. EXAMPLES: MONOCLONAL ANTIBODY C2B8 INHIBITS THE IMMUNE RESPONSE AGAINST IMMUNOTOXIN BR96 sFv-PE40

6.1 THE MAJORITY OF PATIENTS RECEIVING BR96 sFv-PE40 DEVELOP ANTI-TOXIN ANTIBODIES

BR96 sFv-PE40 is a single chain immunotoxin comprised of the variable region of the monoclonal antibody BR96, which recognizes the Le^y antigen present on a number of carcinomas and a toxin portion that is a 40 kD form of *Pseudomonas* exotoxin lacking its cell-binding domain. BR96 sFv-PE40 has antitumor activity *in vivo* (*see, e.g.*, Friedman *et al.*, 1993, J. Immunol.150:3054-3061), but its efficacy in humans is potentially limited by the immune response mounted against the toxin component of the molecule.

In a phase I dose escalation study, a total of 46 patients were treated with BR96 sFv-PE40 over 9 dose levels on a four dose, twice-weekly schedule in 28 day cycles. Serum samples were obtained from the patients at various time points during the course of treatment with BR96 sFv-PE40. The presence of human anti-toxin antibodies (HATA) in the serum samples was quantitated by the following ELISA procedure:

Immulon II ELISA plates were coated with 100 µl Capture Reagent (2µg/mL of BR96 sFv-PE40 in PBS/0.1% NaN_3) per well. The plates were covered with plastic sealers and incubated overnight at 4°C. The plates were washed with PBST (PBS (pH 7.4, 10 mM phosphate, 2.7 mM KCl, and 137 mM NaCl)/0.05% Tween-20/0.1% NaN_3). 200 µL Blocking Buffer (1.0% gelatin/3.0% powdered milk in 100 mL PBS) was added and the plates covered and incubated for 1-2 hours at 37°C. The plates were then washed 3 times with PBST. Reference standard (BR96 sFv-PE40 hyperimmunized cynomolgus monkey sera) and various dilutions of the patients' sera were added to the plates, the plates were covered and incubated for 1-2 hours at 37°C. The plates were washed 4 times with PBST then the secondary antibody cocktail (1:5000/1:2500/1:2500 dilution in binding buffer of goat anti-human IgG/IgM/IgA, respectively) was added. The plates were covered and incubated for 1-2 hours at 37°C. Following incubation, the plates were washed 5 times with PBST. Detection reagent (1 mg/mL PNPP (4-Nitrophenyl phosphate) in DEA (diethanolamine) buffer) was added to the plates and the plates covered and incubated for 25 minutes at 25°C, after which time 50 µL 3N NaOH stop reagent was added to the plates. The optical density (OD) of the ELISA signal was measured at 450 nm and the greatest serum dilution that gave rise to a detectable signal, an indirect measure of HATA concentration in the serum, was determined. Conversion to seropositivity is defined as an OD of three times the standard deviation (SD) above background OD. A titer of 1:7290 is the cut off for serologic significance.

The majority of patients (71%) developed a significant (*i.e.*, 1:7290 or greater) human anti-toxin antibody (HATA) response directed against the toxin moiety of BR96 sFv-PE40 by Day 22 of the first course (Table 1).

TABLE 1

Course	Day 1	Day 4	Day 8	Day 11	Day 15	Day 22
Course 1 *No. of patients tested with HATA titer > 1:7290*	4/41	6/41	7/38	16/39	18/36	25/35
% of patients tested with HATA titer > 1:7290	9.8%	14.6%	18.4%	41%	50%	71%
Course 2 *No. of patients tested with HATA titer > 1:7290*	22/41	18/23	17/22	16/21	19/22	19/19
% of patients tested with HATA titer > 1:7290	53.7%	78.3%	77.3%	76.2%	86.4%	100%

Table 1: HATA frequencies after 2x/wk administration of BR96 sFv-PE40

6.2 HATA TITERS OF GREATER THAN 1:20,000 LIMIT THE EFFICACY OF BR96 sFv-PE40

6.2.1 HATA TITERS OF GREATER THAN 1:20,000 ARE NEUTRALIZING

Next, the effect of the presence of HATA on the cytotoxicity of BR96 sFv-PE40 was determined. Human tumor cell lines were exposed to serum from patients to whom BR96 sFv-PE40 had been administered, and the cytotoxic effect of the serum (attributable to BR96 sFv-PE40 activity), reflected as an inhibition of protein synthesis in the tumor cell lines, was measured essentially as described in Friedman et al., 1993, J. Immunol. 150:3054-61.

L2987 cells were cultured as monolayers at 37°C/5% CO_2 in RPMI 1640 supplemented with 10% FBS and 50 U/ml penicillin/streptomycin. Tumor cells were plated onto 96-well flat bottom tissue culture plates (1 x 10^4 cell/well) and kept at 37° for 16h. The patients' serum samples were serially diluted in growth media and 0.1 ml added to wells containing the L2987 cells for 45h at 37°C. As a control, BR96 sFv-PE40 diluted in growth media to a concentration of 10 ng/ml was added to wells of L2987 cells for 45h at 37°C in parallel with the serum samples. Each dilution was done in triplicate. The cells were pulsed with [^{3}H]-leucine (1μCi/well) for an additional 3h at 37°C. The cells were lysed by freeze-thawing and harvested in a cell harvester. Incorporation of [^{3}H]-leucine into

cellular protein was determined using a liquid scintillation counter. The percentage inhibition of protein synthesis was determined relative to untreated cells.

The results of a representative cytotoxicity assay are depicted in FIG. 1. FIG. 1 shows the percentage inhibition protein synthesis elicited by serum obtained from (i) patient 1-003 at day 4 of treatment with BR96 sFv-PE40, when the HATA titer was 1:21870; (ii) patient 1-003 at day 22 of treatment with BR96 sFv-PE40, when the HATA titer was 1:590490; and (iii) patient 1-005 at day 4 of treatment with BR96 sFv-PE40, when the HATA titer was 1:90. As a positive control, 10 ng/ml BR96 sFv-PE40 was used. The sample from patient 1-005 with a HATA titer of 1:90 had equivalent inhibition of protein synthesis to the BR96-PE40 control (>60%). In contrast, the day-4 sample from patient 1-003 had a slight reduction of protein synthesis inhibition and the day-22 sample reduced the protein synthesis inhibition until the sample had been diluted 100,000 fold (FIG. 1). Serum samples with HATA titers of greater than approximately 1:20,000 were found to be neutralizing in the cytotoxicity assay (FIG. 1).

6.2.2 HATA TITERS OF GREATER THAN 1:7,290 RESULT IN RAPID CLEARANCE OF BR96 sFv-PE40

To analyze the pharmacokinetics ("PK") of the BR96 sFv-PE40 immunotoxin, *i.e.*, to determine BR96 sFv-PE40 clearance from the patient's circulation, quantitative ELISA experiments were conducted to determine the amount of BR96 sFv-PE40 in the patients' sera at various time points during the treatment course. All patients in the PK cohort received a BR96 sFv-PE40 dose of 0.641 mg/m^2. Sampling time points were immediately prior to administration of BR96 sFv-PE40, and, then as indicated following administration of BR96 sFv-PE40, at post infusion 5, 15, 30, 60, 90, 180 and/or 240 minutes.

The ELISA experiments were conducted as follows. Anti-PE monoclonal antibody EXA2-1H8 was diluted in sterile PBS (10 mM phosphate, 2.7 mM KCl, and 137 mM NaCl at pH 7.4) to yield a 4 μg/mL working solution (Siegall *et al.*, 1994, J. Immunol. 152(5):2377-84). The ELISA plates were prepared plates by adding 100 μL of the working solution of EXA2-1H8 to each well. The assay plates were then covered with a plate sealer and stored at 4°C for 16-48 hours. The coating solution was manually flicked from the plates. Next, 200 μL of blocking buffer (PBS/0.05% Tween-20/3.0% bovine serum albumin) was added to each well. The plates covered and during a 1-2 hour incubation at room temperature in blocking buffer. The plates were then washed three times with PBST wash buffer. 100 μL of the diluted reference standards (3.2, 2.4, 1.6, 0.8, 0.4, 0.2, and 0.1 ng/mL) or study samples (*i.e.*, patients' sera) added to their appropriate wells. The plates

were covered and incubated for 2 hours. The plates were then washed four times with PBST. 100 µL of the biotinylated BR96 idiotypic monoclonal antibody 757-4-1 (Siegall *et al.*, 1994, J. Immunol. 152(5):2377-84) was added to each well at a concentration of 80 ng/ml. The plates were covered and incubated for one hour, then washed four times with PBST. 100 µL of diluted streptavidin-HRP conjugate (1:5,000) was added to each well, and the plates incubated, covered, for 30 minutes. The plates were then washed 5 times with PBST, 100 µL of TMB buffered substrate (Sigma, St. Louis, Missouri) added to each well, and the plates incubated, uncovered, for up to 10 minutes. 100 µL of 1M H_3PO_4 was added to each well, the plates shaken approximately 30 seconds and the optical density measured within one hour of developing the colorimetric signal.

The mean peak plasma concentration was 413ng/mL ± 101 and the calculated mean plasma half life (T½) was 2.5 hours. The corresponding HATA titers are listed and demonstrate a clear relationship between increasing titers and more rapid clearance of the drug (BR96 sFv-PE40) from the circulation (Table 2).

TABLE 2

TIME	2-010	2-011	2-012	1-023	1-024	1-026	1-028	1-029
C1D1	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml
HATA Titer	1:270	1:30	1:30	1:270	1:10	1:10	1:90	1:10
Pre	0	0	8	3	0	0	15	0
Post	304	298	180	367	421	476	453	284
5 Min	272	405	310	374	439	535	458	244
15 Min	259	276	272	338	400	435	438	238
30 Min	264	276	285	316	384	399	391	211
60 Min	256	253	251	303	323	379	322	206
90 Min	214	192	208	230	285	280	268	169
120 Min	200	168	187	196	258	261	257	150
180 Min	155	140	151	168	193	130	203	122
240 Min	125	101	118	120	146	126	164	111
C1D4								
HATA Titer	1:810	1:30	1:30	1:90	1:90	1:810	1:90	1:10
Pre	5	1	6 l	3	0	0	18	0
Post	373	330	272	447	524	550	336	279
30 Min	300	305	198	408	416	457	363	297
120 Min	201	179	157	285	240	276	229	228
C1D8								
HATA Titer	1:810	1:30	1:30	1:810	1:270	1:270	1:270	1:10
Pre	0	1	4	2	0	0	7	0.3
Post	407	368	163	347	273	320	386	NA
30 Min	267	262	263	352	254	246	307	NA
120 Min	186	197	212	265	280	143	160	NA
C1D11								
HATA Titer	1:590490	1:90	1:30	1:7290	ND	1:7290	1:2430	ND
Pre	0	0	4	2	0.4	0	15	NA
Post	NA	226	231	79	NA	0	13	NA
5 Min	NA	210	332	67	NA	0.4	10	NA
15 Min	NA	200	245	61	NA	0.4	12	NA
30 Min	NA	181	240	36	NA	0	11	NA
60 Min	NA	163	223	19	NA	0	10	NA
90 Min	NA	143	215	12	NA	0	12	NA
120 Min	NA	140	178	4	NA	0.1	5	NA
180 Min	NA	113	149	2	NA	0	5	NA

240 Min	NA	99	139	1	NA	0.2	5	NA
HATA Titer C1D15	1:177147 0	1:243 0	ND	ND	ND	1:6561 0	1:2187 0	ND
HATA Titer C1D22	1:177147 0	1:243 0	ND	ND	ND	1:2187 0	1:2187 0	ND

Table 2: Pharmacokinetics and HATA Titer Correlation (BR96 sFv-PE40 levels measured in ng/ml)

These data indicate that BR96 sFv-PE40 is rapidly cleared from the circulation of individuals with HATA levels of greater than 1:7,290.

6.3 ADMINISTRATION OF C2B8 INHIBITS THE PATIENTS' IMMUNE RESPONSE TO BR96 sFv-PE40

The anti-CD20 antibody C2B8 (in the form of the drug Rituxan®) was incorporated into the BR96 sFv-PE40 phase I study to evaluate its ability to limit the BR96 sFv-PE40-induced immune response. A cohort of patients was treated with a full course of C2B8 (4 doses of C2B8 at a dose of 375 mg/m^2 over 2 weeks) prior to receiving the first dose of BR96 sFv-PE40. Prior to receiving the first dose of BR96 sFv-PE40, peripheral B cell depletion was documented.

To determine the percentage of peripheral B cell depletion in a patient, 100µl of the patient's blood sample was mixed 5 µl of each of three antibodies, anti-CD19 (a B cell marker), anti-CD20 (another B cell marker) and anti-CD45 (a lymphocyte marker). Each of the antibodies was conjugated to a different fluorescent signal. The antibody-sample mixture was incubated for 15 minutes in the dark. Following the 15-minute incubation period, 2 ml of Saponin-Formalin Solution (a 1:5 dilution of a solution containing 100ml of 10% formalin, 100 ml 10x PBS, 700 mg of Saponin and 2ml of 5% sodium azide) was added to the tubes. After the Saponin-Formalin Solution was added to the blood sample, the resulting mixture was vortexed and incubated for 10 minutes in the dark. Addition of this solution resulted in the simultaneous lysis of red blood cells and partial formation of white cells. The cells were pelleted by a 5 minutes centrifugation at 1500 rpm for 5 minutes, and the supernatant aspirated. The cells were resuspended in a 1x PBS wash solution, and centrifuged as before, and the supernatant aspirated. The resulting pellet was resuspended in 0.2 ml of 1x PBS and subjected to flow cytometric analysis on a

FACScan/FACSort instrument. The percentage of B cells in a sample was a total of anti-CD19 and anti-CD20-derived fluorescence over total fluorescence.

The extent of peripheral B cell depletion is indicated in Table 3.

TABLE 3

B Lymphocyte Counts (%) after 4 doses of C2B8

Patient #	Pre-C2B8 treatment	Post C2B8 treatment	Percentage B cell depletion
1-0036	19%	2%	89.5%
1-0037	9%	<1%	> 89%
1-0038	21%	<1%	> 95%

Patients were then given BR96 sFv-PE40 three times a week for two weeks followed by a 14-day interval rest period (1 course = 4 weeks). Serum samples were obtained from the patients prior to each dose to measure the titer of anti-toxin antibodies (HATA).

The first three patients treated on the three-times weekly schedule did not receive prior treatment with C2B8 and all developed HATA titers of greater than 1:7,290 by Day 22 of Course 1 (Table 4). In contrast, none of the patients pre-treated with C2B8 developed significant HATA titers by day 22 of the course (Table 4). More importantly, the absence of a significant HATA titer in this cohort of patients has been maintained through additional cycles of therapy with BR96 sFv-PE40 (Table 4), with one patient receiving four full courses.

TABLE 4

	Patient #	Course#	Day 1	Day 3	Day 5	Day 8	Day 10	Day 12	Day 15	Day 22	Day 29
No C2B8	1-0033	1	1:30	1:30	1:30	1:30	1:30	1:30	1:810	1:21,870	1:21,870
		2	1:2430	1:2430	1:2430	1:7290	1:7290	1:7290	1:7290	1:7290	1:7290
		3	1:7290	1:7290	1:7290	1:65,610	1:65,610	1:196,830	1:65,610	1:196,830	NA
	1-0034	1	1:90	1:30	1:30	1:30	1:30	1:90	1:810	1:7290	1:7290
	1-0035	1	1:270	1:270	1:270	1:270	1:810	1:810	1:7290	1:7290	1:7290
		2	1:7290	1:7290	1:7290	1:7290	1:7290	1:7290	1:7290	1:7290	1:7290

C2B8 Pre-treatment											
	1-0036	1	1:30	1:30	1:30	1:30	1:30	1:90	1:90	1:90	1:90
		2	1:90	1:30	1:30	1:90	1:30	1:90	1:30	1:90	1:90
	1-0037	1	1:270	1:270	1:90	1:30	1:30	1:30	1:90	1:2430	NA
		2	1:810	1:30	1:270	1:270	1:810	1:270	1:810	1:2430	1:2430
		3	1:810	1:810	1:810	1:810	1:810	1:2430	1:2430	1:7290	1:7290
		4	1:7290	1:2430	1:2430	1:810	1:2430	1:2430	1:7290	1:65610	NA
	1-0038	1	1:30	1:90	1:90	1:30	1:30	1:30	1:10	1:10	1:10
	4-0001	1	1:30	1:30	1:10	1:10	1:30	1:10	1:30	NA	NA
		2	1:30	1:30	1:30	1:30	1:270				

Table 4: Measurement of HATA in patients over 3 courses of immunotherapy with BR96 sFv-PE40 on a 3x/wk Schedule. HATA measurement was conducted as described in Example 6.1, *supra*. NA - Not Available.

The results in Table 4 demonstrate that depletion of circulating B-cells with an anti-CD20 antibody, such as C2B8, can effectively delay and/or prevent the development of a humoral response to an immunogenic molecule.

7. SPECIFIC EMBODIMENTS, CITATION OF REFERENCES

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various references, including patent applications, patents, and scientific publications, are cited herein, the disclosures of which are incorporated herein by reference in their entireties.

WHAT IS CLAIMED IS:

1. A method for reduction or prevention of an immune response to a toxin in a patient, comprising administering to a patient an anti-CD20 monoclonal antibody in an amount sufficient to deplete the patient's circulating B-cells, wherein said anti-CD20 monoclonal antibody is administered to the patient prior to, concurrently with or after the administration of the toxin to the patient.

2. The method of claim 1, wherein the toxin is a component of a fusion protein comprising the toxin and a second protein.

3. The method of claim 1, wherein the toxin is a component of a conjugate comprising the toxin and a second protein.

4. The method of claim 1, wherein the toxin is abrin, ricin A, *Pseudomonas* exotoxin, *Diphtheria* toxin, *Clostridium baratii* type F-like toxin, *Clostridium butyricum* type E-like toxin, botulinum toxin, tetanus toxin, modeccin, bungarotoxin, shigatoxin, cholera toxin, bryodin, saporin or gelonin.

5. The method of claim 2, wherein the fusion protein is denileukin diftitox.

6. The method of claim 2, wherein the fusion protein is BR96 sFv-PE40.

7. The method of claim 1, wherein the anti-CD20 monoclonal antibody is a chimeric antibody.

8. The method of claim 7, wherein the chimeric anti-CD20 antibody is chimeric 2B8 antibody.

9. The method of claim 7, wherein the chimeric anti-CD20 antibody is chimeric B1 antibody.

10. The method of claim 1, wherein the anti-CD20 monoclonal antibody is a humanized antibody.

11. The method of claim 1, wherein the anti-CD20 monoclonal antibody is a human antibody.

12. The method of claim 1, wherein the amount is sufficient to deplete the patient's circulating B-cells by at least 25%.

13. The method of claim 12, wherein the amount is sufficient to deplete the patient's circulating B-cells by at least 35%.

14. The method of claim 13, wherein the amount is sufficient to deplete the patient's circulating B-cells by at least 50%.

15. The method of claim 14, wherein the amount is sufficient to deplete the patient's circulating B-cells by at least 60%.

16. The method of claim 15, wherein the amount is sufficient to deplete the patient's circulating B-cells by at least 75%.

17. The method of claim 16, wherein the amount is sufficient to deplete the patient's circulating B-cells by at least 85%.

18. The method of claim 17, wherein the amount is sufficient to deplete the patient's circulating B-cells by at least 90%.

19. The method of claim 12, 13, 14, 15, 16, 17, or 18, wherein said depletion is achieved prior to the administration of the immunogenic molecule to the patient.

20. The method of claim 1, wherein the anti-CD20 monoclonal antibody is administered concurrently with the toxin.

21. The method of claim 1, wherein the anti-CD20 monoclonal antibody is administered prior to administration of the toxin.

22. The method of claim 21, wherein the toxin is administered within two weeks of administration of the anti-CD20 monoclonal antibody.

23. The method of claim 21, wherein the toxin is administered within ten days of administration of the anti-CD20 monoclonal antibody.

24. The method of claim 21, wherein the toxin is administered within one week of administration of the anti-CD20 monoclonal antibody.

25. The method of claim 21, wherein the toxin is administered within five days of administration of the anti-CD20 monoclonal antibody.

26. The method of claim 21, wherein the toxin is administered within three days of administration of the anti-CD20 monoclonal antibody.

27. The method of claim 21, wherein the toxin is administered within two days of administration of the anti-CD20 monoclonal antibody.

28. A kit comprising in one or more containers a purified anti-CD20 antibody and a purified toxin.

29. The kit of claim 28, wherein the anti-CD20 monoclonal antibody is chimeric 2B8.

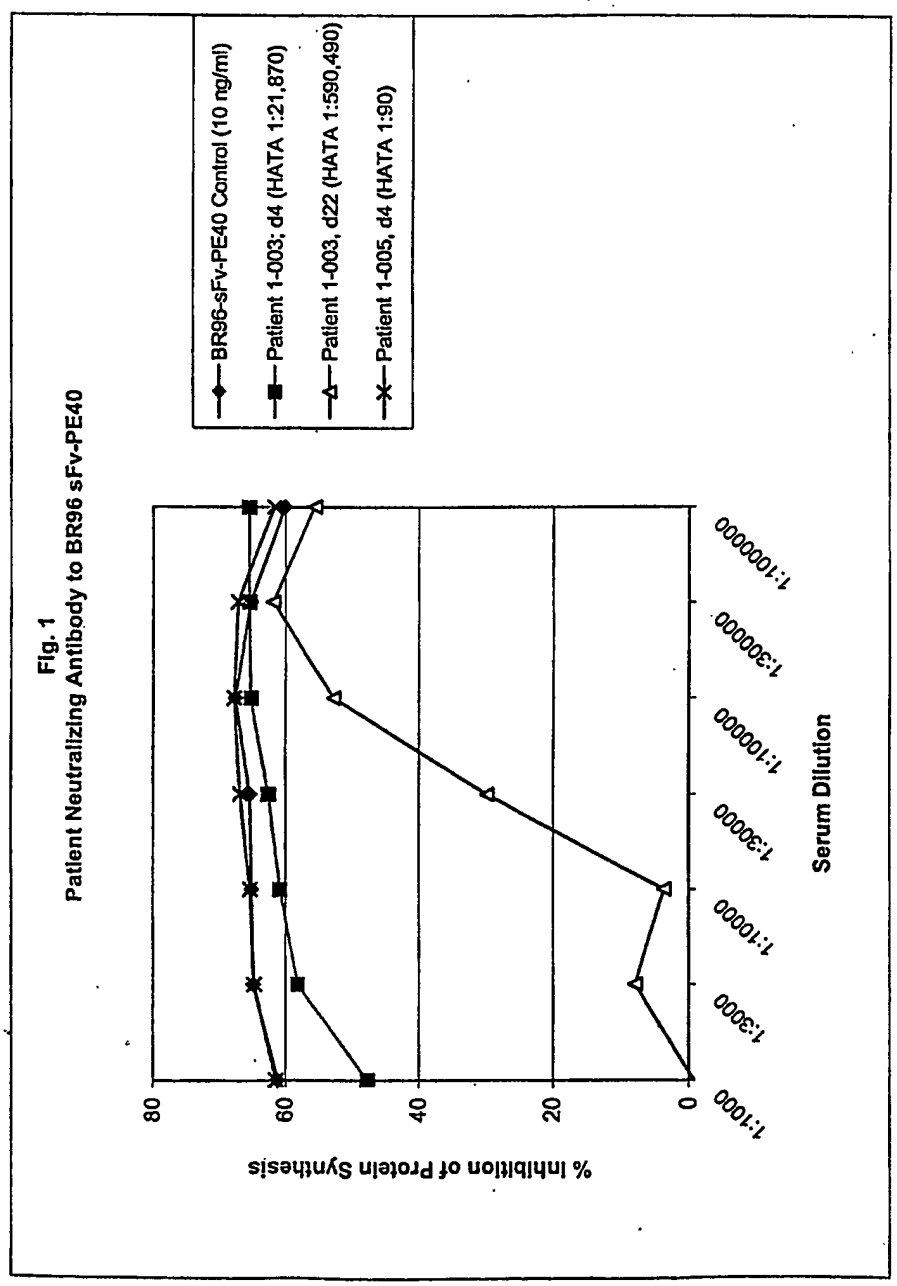
Fig. 1
Patient Neutralizing Antibody to BR96 sFv-PE40
BR96-sFv-PE40 Control (10 ng/ml)
Patient 1-003; d4 (HATA 1:21,870)
Patient 1-003, d22 (HATA 1:590,490)
Patient 1-005, d4 (HATA 1:90)
% Inhibition of Protein Synthesis
0
20
40
60
80
1:1000
1:3000
1:10000
1:30000
1:100000
Serum Dilution

9632-015

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/14600

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 39/395; C07K 16/28, 16/46
US CL : 424/143.1, 133.1, 134.1, 144.1, 153.1, 173.1, 178.1, 183.1; 530/387.3, 388.22, 388.7, 388.73, 391.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/143.1, 133.1, 134.1, 144.1, 153.1, 173.1, 178.1, 183.1; 530/387.3, 388.22, 388.7, 388.73, 391.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GONZALEZ-STAWINSKI et al. Hapten-Induced Primary and Memory Humoral Responses are Inhibited by the Infusion of Anti-CD20 Monoclonal Antibody (IDEC-C2B8 Rituximab). Clinical Immunology. February 2001, Vol. 98, No. 2, pages 175-179, see entire document.	1-29
Y	PAI et al. Immunotoxin Therapy for Cancer. JAMA. 06 January 1993, Vol. 269, No. 1, pages 78-81, see entire document.	1-29

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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INTERNATIONAL SEARCH REPORT

PCT/US02/14600

Continuation of B. FIELDS SEARCHED Item 3:
WEST 2.1, MEDICINE,BIOTECH (compendium databases on DIALOG) search terms: inventor names, cd20, anticd20, rituxan, B1, 2B8, chimeric, immunotoxin, toler?, blocks, delet?, B cells, autoimmun?